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Flexible membrane proteins: functional dynamics captured by mass spectrometry

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Membrane proteins are flexible molecular machines, responsible for the exchange of molecules in and out of the cell, which have evolved to perform specific and complex tasks with great efficiency. Obtaining accurate descriptions of their dynamics in the context of their function represents a major challenge for structural biology. Here we chart recent developments in mass spectrometry designed to characterize changes in the dynamics of membrane proteins in response to ligand binding or post-translational modifications. We focus on cooperative movements and structural changes across a range of timescales, from milliseconds to minutes, and highlight the contributions of mass spectrometry to our understanding of molecular mechanisms of diverse transmembrane processes.

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Introduction

Biological membranes delimitate the cellular entity and consist of phospholipid bilayers in which proteins are embedded [1]. Membrane proteins mediate much of the function of the cell, including energy conversion, transport, signaling and recognition [2]. As such it is not surprising that membrane proteins comprise more than half of all known drug targets [3]. Uncovering the molecular mechanisms of these proteins in action therefore represents a prominent frontier in modern structural biology.

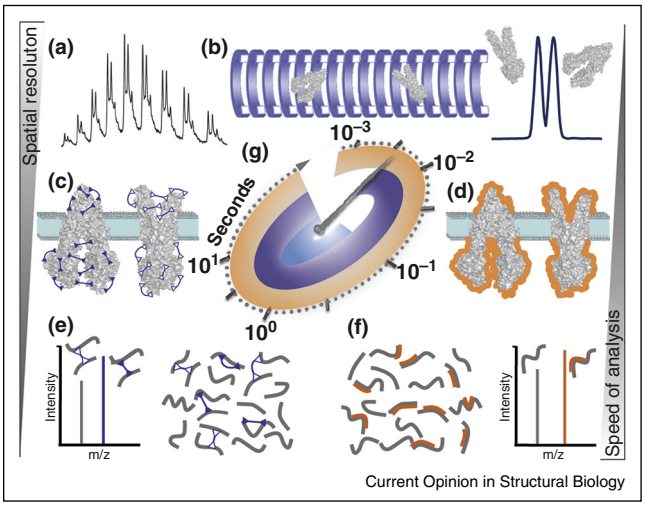
Over the past decades considerable insight has been garnered from high-resolution structural methods that are indispensable to the mechanistic studies of membrane-associated events [4–7]. When applied to membrane proteins these methods are often hampered due to the low solubility and stability of these proteins once extracted from the natural lipid environment. Equally challenging is the characterization of their flexibility and unique motions. These movements often underlie the function and regulation of transmembrane processes and span a range of timescales from milliseconds to minutes [8–10]. Moreover, membrane proteins frequently undergo dynamic interactions and constant reorganization within the fluidic biological membrane [11,12], forming heterogeneous macromolecular assemblies that are often beyond the scope of traditional structural biology approaches such as X-ray diffraction and NMR.

The gap between the biomedical significance and existing structural knowledge of membrane protein dynamics has motivated new experimental approaches [13–17]. Among them is mass spectrometry. In recent years mass spectrometry based methods have emerged as alternative means to characterize biological heterogeneity and dynamics with temporal resolution down to the millisecond scale [18–23]. Moreover the breakthrough in MS for studying membrane protein complexes, delivered directly from micellar solutions, is now paving the way for the elucidation of composition, stoichiometry and interactions of integral and peripheral membrane protein assemblies [24–28]. Here we discuss recent advances in mass spectrometric approaches pinpointing membrane protein motions that span over six orders of magnitude, from submillisecond to seconds or longer (Figure 1), and summarize novel insights that have been gained on the functional dynamics of membrane-embedded assemblies.

Capturing rapid structural fluctuations by chemical labeling mass spectrometry

The marriage of chemical labeling techniques and mass spectrometry some years previously has enabled the elucidation of conformational change and dynamics of soluble proteins [15,29–31]. Its applicability to membrane proteins however has only been demonstrated recently, primarily due to the practical difficulties associated with labeling reagents penetrating the detergent micelle or membrane bilayer. Chemical labeling experiments fall into two broad categories: labile modification, for example, isotopic exchange, and permanent modification introduced by covalent labeling such as hydroxyl radical footprinting. Both are capable of exploring rapid structural fluctuations with labeling reactions taking place on a millisecond timescale [32,33]. The former, represented by hydrogen-deuterium exchange mass spectrometry

Figure 1



Mass spectrometry approaches designed to study membrane protein dynamics. Membrane protein complexes can be transferred intact into the gas phase of the mass spectrometer allowing direct interrogation of small molecule binding **(a)** and simultaneous monitoring of the resultant conformational changes by ion mobility **(b)**. Alternatively, comparative cross linking **(c)** or labeling techniques **(d)** can be employed to capture the dynamic behavior of membrane proteins before enzymatic cleavage and subsequent MS analysis **(e)** and **(f)**. In (c) and (e) light and heavy cross linkers are denoted with empty and filled arrows respectively. **(g)** Temporal resolution of different MS methods is indicated for the labeling techniques (orange), ion mobility mass spectrometry (blue) and crosslinking (light blue). Sidebars represent trends toward increased spatial resolution and speed of analysis.

(HDX-MS), has been employed in several studies for the investigation of the mechanism of activation of G protein-coupled receptors (GPCR) and transmembrane signaling. Application of HDX-MS to these targets has been demonstrated in revealing conformational changes that occur on a millisecond timescale [34–37].

Heterotrimeric G proteins are composed of a nucleotide binding α subunit (Gα) and an obligate dimer of the β and γ subunits (Gβγ) [38,39]. In their inactive form, Gα subunits are bound to GDP and tightly associated with Gβγ. Binding with the receptor activates heterotrimeric G proteins (Gs, the stimulatory G protein for adenylyl cyclase) through promoting the release of GDP, thus initiating a cascade of transmembrane signaling events. Results obtained from HDX-MS experiments complement the high-resolution snapshot provided by the crystal structure and uncover the mechanistic details of receptor-mediated activation. In the case of the β_2 adrenergic receptor (β_2AR) HDX-MS reveals a dramatic increase in exchange rate in the β1 strand. This strand links the second intracellular helix ICL2 of the agonist-bound receptor to the P-loop that coordinates the β-phosphate of GDP in Gαs [35]. Increased flexibility at the receptor-binding surface (β1 strand) destabilizes the local hydrogen-bonding network, and is coupled to conformational changes in the highly conserved P-loop at the nucleotide-binding pocket. Overall therefore the study uncovers an important link between structural dynamics of the two regions and relates receptor binding to GDP dissociation.

Continuing our focus on β_2AR activation but this time with respect to changes in dynamics associated with ligand binding, a marked decrease in flexibility of the intracellular helix ICL2 was revealed through HDX-MS [34]. A range of functionally distinct ligands of β_2AR were screened including a full agonist isoproterenol, a partial agonist clenbuterol, an antagonist alprenolol, and two inverse agonists carazolol and timolol [40]. Results showed that binding of agonists yielded greater protection from exchange than antagonists and inverse agonists. Conformational modulation was observed in regions spanning extracellular to intracellular regions of the receptor. Notably, binding of all five ligands yielded a significant reduction in dynamics in the ICL2 region. In the *apo*β_2AR structure ICL2 is proposed to be highly dynamic adopting either an extended/unstructured [41] or helical form [42]. The HDX data therefore suggest a shift in the conformational equilibrium of ICL2 toward a more rigid helical state of ICL2, priming it for the recruitment of G proteins or other effectors at the intracellular side.

Interestingly for both of the two HDX studies outlined above the dynamic motions involved in β_2AR-Gs activation were found to be on a comparable timescale to the fast amide exchange rate; under these conditions a maximum time resolution of millisecond is therefore achievable. When combined these investigations uncover important insight into the activation mechanism of GPCR-mediated signaling, its inhibition and the structural elements that are responsible for performing and controlling function.

Dynamics play a fundamental role in gating of membrane-embedded channels that are thought to operate via domain movements, again on a millisecond timescale. The dynamic gating mechanism of the potassium channel KirBac3.1 regulates the selective flow of K^+ in response to a variety of stimuli [43]. To probe the molecular mechanism, and to compare the open and closed states of KirBac3.1, hydroxyl radicals were employed to induce oxidation of amino acids located on the surface of protein. In this case the exposure period for oxidative labeling can be as brief as just a few milliseconds in order to capture rapid transitions between the two states. Results were consistent with considerable flexibility and large allosteric changes, including a rotational movement of the pore-lining transmembrane helices, which combine to open the gate. The study thus provides direct evidence for ion channel gating dynamics on a millisecond timescale and pinpoints the helices that move apart at the 'helix-bundle crossing' [44].

While informative the above examples of membrane protein dynamics were determined in detergent micelles. A long-term goal for structural biologists however is to probe these dynamics in the context of the biological membrane, or even on the surface of living cells. In this regard, a covalent labeling method was developed to explore the dynamics of voltage gating of the bacterial porin OmpF *in vivo*, within its natural lipid environment [45]. To do this membrane proteins on the cell surface of a living *Escherichia coli* were covalently modified with hydroxyl radicals generated *in situ*. A total of 19 modifications of OmpF were identified located in the external loop area, β-strands, and periplasmic turning area. Conformational changes associated with OmpF gate closing in response to variations in the transmembrane potential were also characterized. Protection from oxidation was observed for peptides in the β-stranded area, deep inside the porin channel upon the transition from open to closed states. Rather unexpectedly, the results point to flexibility of the β-barrel structure, previously considered to be rather rigid, but interestingly, crucial for porin gating under physiological conditions.

Charting conformational flexibility with ion mobility

Recent discovery of MS conditions for the release of membrane protein assemblies, encapsulated in detergent micelles, into the vacuum of a mass spectrometer offers a new phase for their study [46]. By exploiting the protective effect of detergent micelles in the gas phase, noncovalent interactions of membrane protein complexes are preserved. It is therefore not only possible to explore the subunit composition, stoichiometry and interactions, but more importantly, to assess the effects of lipids, nucleotides, and drugs on intact membrane assemblies [25,26,47]. For instance, this approach has allowed study of the membrane import of the bacterial cytotoxin ColE9 [48••]. It is established that ColE9 recruits the outer-membrane porin OmpF by threading its intrinsically unstructured translocation domain, forming a transient translocon complex. Using MS a mechanism was unraveled by which the unstructured region of ColE9 is able to thread back for a second time through OmpF, anchoring its highly flexible N-terminal tail via two OmpF attachment points.

As gas phase excitation is required to release membrane protein complexes from detergent micelles, one of the prominent questions is whether the collision with gas molecules would cause structural distortion of the ions. To address this ion mobility (IM), a technique that probes the topology of gaseous ions, was employed to assess the conformation of a series of membrane embedded complexes [26,49]. In parallel with these investigations mechanistic studies have shown that release of detergent competes with structural collapse for the internal energy of the ions, yielding 'naked' ions that retain their native-like conformations, at least within the millisecond timescale of the IM measurement [50•]. Very recently new detergents have been reported that enable preservation of native-like structures for a range of protein complexes enabling unfolding experiments to be employed to assess their stability in response to ligand binding [51••].

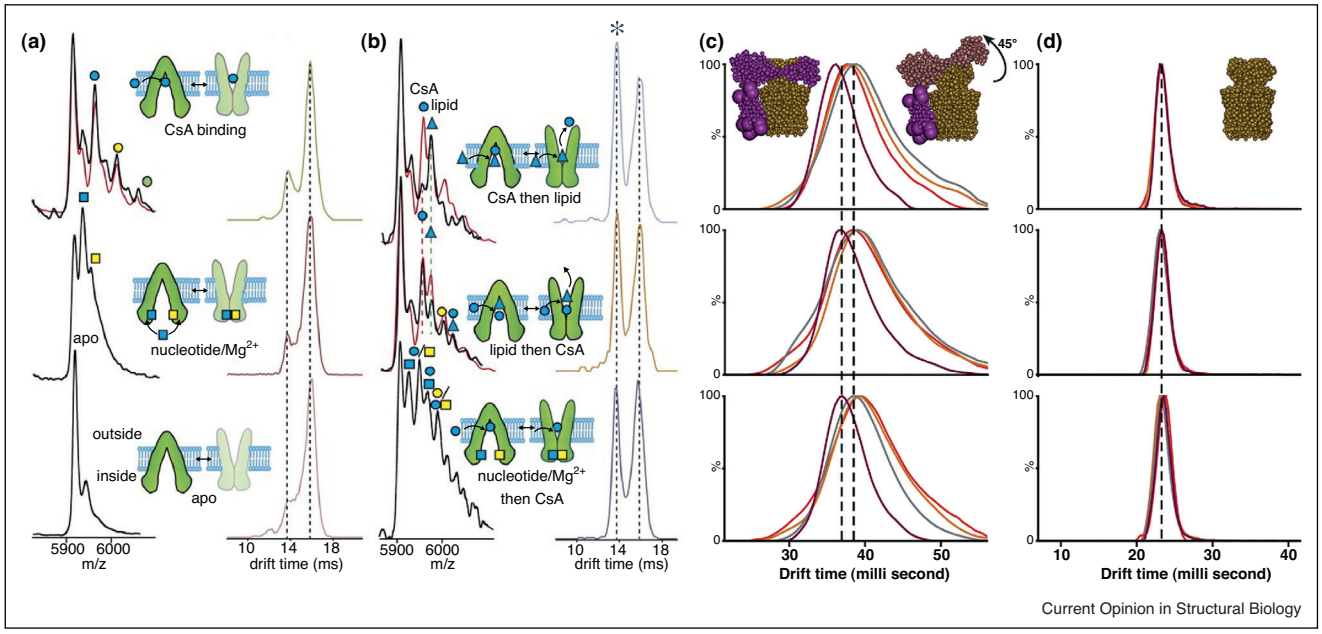
Devoid of detergents and lipids that bind non-specifically, membrane protein ions can be separated according to their gas phase mobility, that is, their ability to migrate through a gas filled chamber [52]. These ion mobility measurements report on the orientationally averaged collision cross sections (CCS), a characteristic parameter of an ion related to its topological contour [53]. A particularly exciting prospect for membrane protein complexes is the coupling of ion mobility with MS to allow conformational and compositional heterogeneity to be recorded simultaneously. This has been recently illustrated in the study of P-glycoprotein (P-gp) [54••].

P-gp is a member of the ATP-Binding Cassette (ABC) family of transporters using ATP hydrolysis to actively export a wide variety of hydrophobic cytotoxic compounds as well as lipids and peptides. The membrane embedded efflux pump is responsible for multidrug resistance in chemotherapies [55]; structurally it consists of two transmembrane domains (TMDs) for substrate binding and two nucleotide-binding domains (NBDs) for ATP hydrolysis [56]. Powered by ATP, the consensus view is that P-gp alternates between its inward-facing and outward-facing conformations to export small molecules [57,58]. The precise gating mechanism however is not well understood, particularly with respect to the transition between inward states and outward states in response to small molecule binding.

A dynamic picture of the conformational changes underlying the potential export mechanism of P-gp was obtained recently using ion mobility MS [54••]. By projecting intact complexes of P-gp lipid, nucleotide and drugs into the gas phase specific interactions with a variety of substrates were preserved after removal of detergent micelles. This enabled their binding rates, stoichiometry and relative affinities to be determined. Moreover, it was possible to monitor the binding of drug molecules against a background of detergents and lipids, and in the presence and absence of ATP (Figure 2a). The investigation revealed an important link between substrate binding and ATPase activity and, furthermore how drug export could be modulated by specific lipid binding.

Importantly, migration of P-gp ions through the mobility chamber results in two well-defined arrival time distributions (ATD, Figure 2b). The difference in CCS values between these two peaks correlates with that calculated for crystal structures of a P-gp homolog, MsbA, captured

Figure 2



Functional dynamics of membrane proteins captured by IM-MS. IM-MS allows simultaneous observation of small molecule binding to P-gp using MS (left panel) and concomitant conformational changes via IM (right panels) **(a)**. Synergetic effects of lipids, nucleotides and drugs result in a greater population of P-gp in the outward-facing form as evident by the increase in the faster ATD peak, labeled * **(b)**. In contrast to data for P-gp unimodal mobility distributions were recorded for the transmembrane domain V_o of *Tt*ATPase with and without the stator subunit I (**c** and **d** respectively). The distinct broadness of V_o pinpoints dynamic motion of subunit I, further explored in series of *in vivo* and *in vitro* solution conditions. A potential role for ATP in maintaining the native conformation of I, by reducing the structural dynamics of V_o, (purple line in c) was proposed based on these results.

in inward-facing and outward-facing forms [57]. Although structures of P-gp trapped in its outward-facing conformation are not yet available, IM analysis suggests that the protein exists in equilibrium between these two states. In its apo-form P-gp was found to adopt predominantly an open, inward-facing conformation. Association of substrates and nucleotides alone did not perturb the equilibrium significantly however concomitant binding of nucleotides, lipids, or drugs induced a significant shift in the equilibrium toward the outward-facing conformer with a smaller CCS. The changes in the equilibrium population were investigated on a timescale of seconds. This is considerably slower than the aforementioned channel gating that occurs within milliseconds. The results therefore highlight structural dynamics of the flexible P-gp that underlie the allosteric catalytic cycle.

In the light of these results for P-gp it is interesting to consider an earlier study where in the dynamics of the inward-facing and outward-facing forms of a related ABC transporter, BmrA, were investigated by means of HDX-MS [59••]. Distinct conformational states were evident from the difference in exchange behaviors and substantial flexibility was identified for the intracellular domains in the open inward-facing state. Presumably these flexible intracellular domains are poised for the transition to the outward facing states upon substrate binding.

The timescale of a typical IM experiment lies in the range of several milliseconds [60], sufficient to characterize the conformational transitions of P-gp which are thought to take place over seconds. Functional dynamics of membrane proteins involving large domain movements rather than major conformational changes however are expected to take place on a millisecond timescale [61], comparable to that of the IM measurement [60]. The rate of rotary catalysis of F-type or V-type ATPases for instance is estimated to be in the range of 10^1–10^2 ms [62,63]. The applicability of ion mobility MS to probe conformational fluctuations linked to rotary catalysis has been demonstrated recently for a bacterial V-type ATPase [64•].

Rotary ATPases are nano-scale engines that operate with remarkably high efficiency, utilizing the energy derived from ATP hydrolysis to pump protons across the membrane or *vice versa* [65]. Structurally they consist of a water-soluble catalytic head and an ion-translocating membrane-embedded base. V-type ATPases undergo reversible assembly/disassembly *in vivo* although the precise triggers for this event are unknown [66]. Gaseous

ions generated from the intact V-ATPase from *Thermus thermophilus* (*Tt*ATPase), together with its subcomplexes formed in solution, were allowed to drift through the ion mobility chamber. ATDs were recorded for each species resolved by mass spectrometry. Unexpectedly, rather than a series of discrete ATD peaks, unimodal Gaussian distributions of varying widths were observed. The diversity of these peak widths indicates that the species populate either one uniform conformation (sharp peak) or a continuum of inter-converting states (broad peaks), occurring on a comparable timescale as the IM measurement if not shorter. Important conclusions can therefore be drawn from these IM-MS experiments. First, CCS distribution recorded for the intact complex (V_1V_o) spans a wider range than would be expected for a single conformer, presumably reflecting conformational dynamics captured for the intact particle along the millisecond time axis of rotary catalysis. Second, by comparing variations in CCS, calculated for different subcomplexes, enhanced flexibility can be assigned to specific subunits, that is, the peripheral stalks in V_1 and subunit I in V_o. Interestingly, the former is mirrored by distinct crystal structures reported previously [67••] and is closely reflected by *in vacuo* molecular dynamics simulations.

The remarkable dynamics of the subunit I were further explored in the context of a series of *in vivo* and *in vitro* conditions. It was found that incubation with ATP resulted in sharper mobility peaks for V_o with shorter drift times, indicative of reduced structural dynamics (Figure 2c). Computational modeling, based on a mosaic of structural models fitted into the V_o density established that in the absence of ATP the distal end of subunit I is free to flex away from the membrane plane by a distance of 10 Å with an angular freedom of 60°. Binding of ATP constrains this dynamic motion, restoring the conformation of I poised for re-association with the V_1 domain.

This structural flexibility of the different parts of the *Tt*ATPase, captured *in vitro*, has significant implications for its operation and regulation *in vivo*. First, the peripheral stalks and subunit I interact in the intact *Tt*ATPase comprising part of the stator component of the rotary bioengine. This component is responsible for counteracting the torque generated by conformational changes associated with ATP hydrolysis/synthesis in V_1 and spinning of the rotor within the membrane. Elasticity is therefore crucial for stabilization and cooperation of the two motors, accommodating a characteristic 'wobbling' of the catalytic head [67••]. In accord, with this proposal, the relatively broad Gaussian ATD recorded is indicative of conformational heterogeneity in the intact ATPase. Second, disassembly of the intact complex, in response to ATP depletion under unfavorable conditions, leads to formation of isolated V_1 and V_o domains (Figure 3a). Once separated in this way the unconstrained domains exhibit intrinsic flexibility of the peripheral stalks and subunit I, as evidenced by conformational broadening of ATDs recorded for V_1 and V_o, respectively.

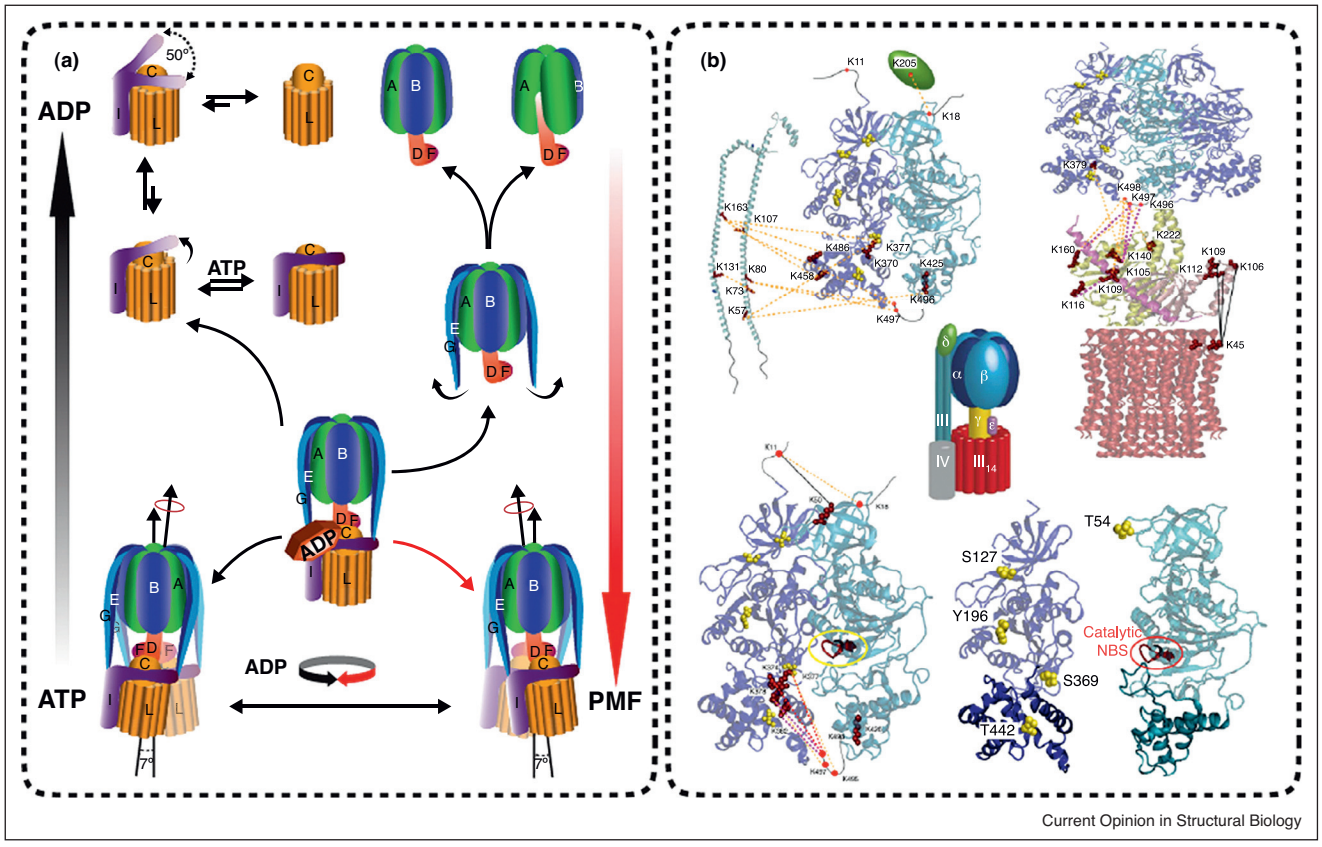
These insights gained from IM-MS led to the proposal of new mechanism in V-ATPase regulation. Substantial fluctuation of the subunit I would likely destabilize its interaction with the rotor ring within the membrane plane, and ultimately trigger its dissociation. Mobility of the surrounding membrane lipids would allow immediate sealing of the resultant gap and subsequently, silencing of the proton flow. Underpinned by nucleotide-modulated flexibility of subunit I this serves to maintain transmembrane proton potential by preventing leakage through decoupled V_o. It is worth noting that binding of ATP to I is able to reverse the process by restoring the active conformation of subunit I, thus priming V_o for its reassembly with V_1 to form the functional V_oV_1 motor.

Quantifying membrane-associated dynamics with comparative cross-linking

Chemical cross-linking combined with mass spectrometry (CXMS) has long been employed for the identification of interaction sites or proximity of proteins, generating inter-residual distance constraints that can be employed in assessing three dimensional structural models of protein complexes [68–71]. The method however is primarily qualitative limiting its application to conformational fluctuations and transient interactions. In attempt to devise some quantitative differences that can be used to assess changes in conformation in response to different stimuli a comparative crosslinking has recently been implemented [72••]. The novel strategy utilizes isotopically labeled cross linkers to quantify the relative intensities of cross linked peptides generated for complexes in different states. An initial study targeting the chloroplast F-ATPase uncovered an important link between phosphorylation, dynamic interactions and regulation of a transmembrane molecular motor.

F-type ATPases or ATP synthases operate in reverse to V-type ATPases by harnessing the membrane potential to synthesize ATP in a manner similar to electric power generation. The chloroplast F-type ATPase (cATPase) operates in the thylakoid membranes of chloroplasts, and unlike the V-type ATPases, contains only one peripheral stalk [73]. Using standard proteomics methods a total of eleven different phosphorylation sites were identified for the intact cATPase freshly isolated from *Spinacia oleracea*. These sites were distributed on stator subunits α, β, δ and II as well as in the rotor subunit ε. To investigate the effects of phosphorylation a phosphotase was introduced to induce *in vitro* dephosphorylation. Comparative cross-linking was then carried out to probe differences in subunit interfaces that exist in phospo and dephospho forms. Significant reduction in inter-subunit crosslinks was observed whereas intra-subunit links remained essentially unchanged. This suggests that dephosphorylation

Figure 3



Translation of membrane protein dynamics into functional regulation of ATPases. **(a)** When the ATP concentration is low in solution binding of ADP to the stator subunit I in the transmembrane V_o domain triggers a conformational change destabilizing its interaction with the peripheral stalks en route to disassembly of the intact complex into V_1 and V_o domains (black arrow). Once unconstrained, by the absence of V_1, remarkable dynamics of subunit I lead to its dissociation and silencing of transmembrane proton flow (upper left). Restoration of ATP levels or likely of the proton motive force (PMF, red arrow) restricts mobility of I priming it for re-assembly. **(b)** A link between phosphorylation and conformational stability has been identified for the chloroplast F-type ATPase (central cartoon). Dephosphorylation was found to induce a conformational change of subunit ε, to reduce interactions between the peripheral stalk and the catalytic head, as well as between α:β subunits in the catalytic head. Decreased inter-subunit cross-links are indicated (dotted lines) and the catalytic nucleotide-binding site in the β subunit is highlighted in red. Structural dynamics are proposed to play an important role in maintaining cATPase in a form that is active toward biogenesis of ATP.

results primarily in changes in dynamic subunit interactions at the quaternary structural level. Moreover, of the 32 inter-subunit crosslinks identified only four were not affected by dephosphorylation. This, together with the stable intra-subunit interactions, implies that conformational changes rather than global destabilization are responsible for the observed decrease in cross-linking. Importantly, projection of the 28 crosslinks that were responsive to dephosphorylation onto a structural model of cATPase revealed their primary location at α:β interfaces in the catalytic head, between subunit I and II in the peripheral stalk, as well as movement of the ε subunit away from the catalytic head (Figure 3b).

Structural consequences of dephosphorylation were also explored by MS analysis of the intact complex. Facile loss of peripheral subunits I, II and δ along with the subunit ε was readily observed implicating a loosening of subunit interfaces in the dephosphorylated state, in accord with the cross-linking results. Moreover, release of nucleotides from the catalytic head was evident from a broad distribution of nucleotide occupancy from zero to three as opposed to a maximum of three in the phosphorylated form. This echoes the reduced contacts between C-terminal domains of α and β subunits established by cross-linking, and points to a regulatory role of phosphorylation in ATP biogenesis. Concerted conformational changes cause opening at the base of the α:β interfaces upon dephosphorylation, and this likely serves to promote nucleotide exchange crucial for catalytic turnover of cATPase. Last but not least, labile association of subunit ε is rationalized by decrease in cross-links of ε to the catalytic head as it contracts from an extended to a compact conformation following dephosphorylation. This

conformational change of ε has been implicated in a few studies maintaining an active rotary motor by preventing stator-rotor attachment [74–76]. Considering the slow rates of phosphorylation/dephosphorylation close to 5 s^{-1} [77] this study serves to illustrate how a combination of MS methods can work together to uncover the structural dynamics of the longer time scales associated with post-translational modifications.

Concluding remarks and outlook

Concomitant with progress in high-resolution structure determination MS based methods arise to offer new possibilities for characterizing membrane protein flexibility. Albeit still in its infancy, the application of ion mobility MS to detergent encapsulated membrane proteins represents a novel strategy to address directly the structural dynamics of membrane-spanning subunits over a wide range of time-scales from milliseconds to hours. The inaccessibility of membrane-buried regions to most chemical probes highlights the search for alternatives to the traditional labeling approach to assess conformational changes and dynamics of these protein assemblies. When accessible however labeling and cross-linking techniques are able to pursue conformational changes within a high-resolution time frame (ms) and with a spatial resolution close to individual amino acid residues.

Besides methodological improvements that continue to extend the reach of mass spectrometry, detergent-free vehicles [48••] including bicelles or nanodiscs in place of micelles, provide a lipid-based environment to support membrane protein malleability and functionality. Laser-induced liquid bead ion desorption (LILBID) [78,79] MS is also finding increasing success in applications with membrane assemblies; while modeling strategies, based on MS restraints, promise to reveal structural features for regions immersed in membrane planes [80]. Embracing these opportunities will help reach the ultimate goal of linking membrane proteomics [81•], high-resolution structure determination and membrane protein dynamics, within the context of lipid bilayer.

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- •• of outstanding interest

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